Carboxypeptidase from *Aspergillus oryzae*

An application to amend the *Australia New Zealand Food Standards Code* with a carboxypeptidase preparation produced by a genetically modified strain of *Aspergillus oryzae*

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EXECUTIVE SUMMARY

The present application seeks to amend Schedule 18—Processing aids of the Australia New Zealand Food Standards Code (the Code) to approve a carboxypeptidase enzyme preparation produced by Novozymes A/S.

Proposed change to Australia New Zealand Food Standards Code – Schedule 18—Processing aids

Schedule 18—Processing aids is proposed to be amended to include a genetically modified strain of *Aspergillus oryzae* expressing a carboxypeptidase from *Aspergillus oryzae* as permitted source for carboxypeptidase.

The application is applied for assessment by the general procedure.

Description of enzyme preparation

The enzyme is a carboxypeptidase D (EC 3.4.16.6), commonly known as carboxypeptidase.

Carboxypeptidases catalyse the hydrolysis of peptide bonds, preferably before C-terminal arginine and lysine residues.

The enzyme is produced by submerged fermentation of an *Aspergillus oryzae* microorganism expressing a carboxypeptidase from *Aspergillus oryzae*.

The carboxypeptidase enzyme preparation is available as a liquid preparation complying with the JECFA recommended purity specifications for food-grade enzymes.

The producing microorganism, *Aspergillus oryzae*, is absent from the commercial enzyme product.

Use of the enzyme

The carboxypeptidase preparation is used as a processing aid in protein hydrolysis of peptide bonds in manufacturing and/or processing of proteins, yeast, and flavouring, the manufacture of bakery products and in brewing. Generally, carboxypeptidase degrade proteins into shorter proteins/peptides and free amino acids.

- In protein hydrolysis of peptide bonds in manufacturing and/or processing of proteins, yeast, and flavouring, the carboxypeptidase is used to hydrolyse proteins into shorter proteins/peptides and free amino acids.

- In the manufacture of bakery products, the carboxypeptidase partially hydrolyses the gluten network in the dough.

- In brewing, the carboxypeptidase increases the amount of free amino nitrogen by hydrolysis of proteins present in the raw materials.

Benefits

The benefits of the action of the carboxypeptidase in protein hydrolysis of peptide bonds in manufacturing and/or processing of proteins, yeast, and flavouring are:

- Use of more mild conditions than the alternative (acid hydrolysis at low pH and high temperature)

- Reduced formation of undesirable side-products (such as 3-monochloropropane-1,2-diol (3-MCPD)) compared with the alternative (acid hydrolysis at low pH and high temperature)

- Reduced formation of salt compared with the alternative (acid hydrolysis at low pH and high temperature)

- Formation of protein hydrolysates with a pleasant taste and minimal bitterness

- Improved protein solubility

- Formation of substances with a pleasant flavour

The benefits of the action of the carboxypeptidase in the manufacture of bakery products are:

- improved dough extensibility

- improved machinability in general and maintain the shape of laminated dough

- more uniform product, shape, surface and colour

The benefits of the action of the carboxypeptidase in brewing are:

- more uniform and predictable fermentation giving less variation caused by batch to batch variations of different raw materials like different malt batches

- increased flexibility in the choice of raw materials such as use of wheat, wheat malt, barley, corn and rice

Safety evaluation

The safety of the production organism and the enzyme product has been thoroughly assessed:

- The production organism has a long history of safe use as production strain for food-grade enzyme preparations and is known not to produce any toxic metabolites.
- The genetic modifications in the production organism are well-characterised and safe and the recombinant DNA is stably integrated into the production organism and unlikely to pose a safety concern.
- The enzyme preparation complies with international specifications ensuring absence of contamination by toxic substances or noxious microorganisms
- Sequence homology assessment to known allergens and toxins shows that oral intake of the carboxypeptidase does not pose food allergenic or toxic concern.
- Two mutagenicity studies *in vitro* showed no evidence of genotoxic potential of the enzyme preparation.
- An oral feeding study in rats for 13-weeks showed that all dose levels were generally well tolerated and no evidence of toxicity.

Conclusion

Based on the Novozymes A/S safety evaluation, we respectfully request the inclusion of the carboxypeptidase in Schedule 18—Processing aids.

INTRODUCTION

The present application describes a carboxypeptidase enzyme preparation produced by submerged fermentation of an *Aspergillus oryzae* microorganism producing a carboxypeptidase from *Aspergillus oryzae*.

The enzyme is a carboxypeptidase D (EC 3.4.16.6), commonly known as carboxypeptidase. The enzyme catalyses the hydrolysis of peptide bonds, preferably before C-terminal arginine and lysine residues.

The carboxypeptidase enzyme preparation is intended to be used as a processing aid in protein hydrolysis of peptide bonds in manufacturing and/or processing of proteins, yeast, and flavouring, in the manufacture of bakery products, and in brewing.

The following sections describe in detail the construction of the genetically modified *Aspergillus oryzae* used as the production organism, the production process, the product specification, the application of the enzyme preparation and finally the safety evaluation of the product including the toxicology program, which has been carried out confirming the safety of the product for its intended use.

The documentation has been elaborated according to the Application Handbook from Food Standards Australia New Zealand as of 1 July 2019, applied as relevant for an enzyme application, i.e. outlining the following section:

- Section 3.1.1 – General requirements
- Section 3.3.2 – Processing aids, subsections A, C, D, E, F

NB! When reading this document it should be noticed that in some reports, the carboxypeptidase enzyme preparation is described as NS PP0083 or by the internal production batch code PPJ55674 and PPJ60566.

CHAPTER 3.1, GENERAL REQUIREMENTS FOR APPLICATIONS

A Executive Summary

An Executive Summary is provided as a separate copy together with this application.

B Applicant details

(a) **Applicant's name/s**

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C Purpose of the application

This application is submitted to provide for amendment of the Australia New Zealand Food Standards Code, Schedule 18—Processing aids to include a genetically modified strain of *Aspergillus oryzae* as permitted source for a carboxypeptidase.

D Justification for the application

The need for the proposed change

Schedule 18—Processing aids contains a list of permitted enzymes of microbial origin. However, Schedule 18—Processing aids does not contain a carboxypeptidase (EC 3.4.16.6) from *Aspergillus oryzae* containing the gene for carboxypeptidase from *Aspergillus oryzae*.

Aspergillus oryzae is an approved host and production strain for a number of enzymes in Schedule 18—Processing aids, including enzymes acing on proteins such as aminopeptidase, asparaginase, aspergillopepsin, metalloproteinase, mucorpepsin, and serine proteinase.

The advantages of the proposed change over the status quo

The carboxypeptidase preparation is used as a processing aid during the processing of protein-containing raw materials. Carboxypeptidases hydrolyse proteins into shorter proteins/peptides, and free amino acids.

The benefits of the action of the carboxypeptidase in protein hydrolysis of peptide bonds in manufacturing and/or processing of proteins, yeast, and flavouring are:

- Use of more mild conditions than the alternative (acid hydrolysis at low pH and high temperature)
- Reduced formation of undesirable side-products (such as 3-monochloropropane-1,2-diol (3-MCPD)) compared with the alternative (acid hydrolysis at low pH and high temperature)
- Reduced formation of salt compared with the alternative (acid hydrolysis at low pH and high temperature)
- Formation of protein hydrolysates with a pleasant taste and minimal bitterness
- Improved protein solubility
- Formation of substances with a pleasant flavour

The benefits of the action of the carboxypeptidase in the manufacture of bakery products are:

- improved dough extensibility
- improved machinability in general and maintain the shape of laminated dough
- more uniform product, shape, surface and colour

The benefits of the action of the carboxypeptidase in brewing are:

- more uniform and predictable fermentation giving less variation caused by batch to batch variations of different raw materials like different malt batches
- increased flexibility in the choice of raw materials such as use of wheat, wheat malt, barley, corn and rice

The benefits, which are described above, are not exclusively obtainable by means of enzyme treatment but can be achieved without the use of enzymes, or with a reduced use of enzymes, through e.g. modified maybe more expensive or less environmentally friendly production processes or recipe changes.

D.1 Regulatory impact information

D.1.1 Costs and benefits of the application

The application is not likely to place costs or regulatory restrictions on industry or consumers. Inclusion of the carboxypeptidase enzyme in Schedule 18—Processing aids will provide the food and beverage industry with the opportunity to improve the processing of proteins during protein hydrolysis of proteins, yeast and flavourings, as well as the manufacture of bakery products and brewing under environmentally friendly and cost efficient production conditions. For the government, the burden is limited to necessary activities for a variation of Schedule 18—Processing aids.

D.1.2 Impact on international trade

The application is not likely to cause impact on international trade.

E Information to support the application

E.1 Data requirements

No public health and safety issues related to the proposed change are foreseen. As outlined in sections 3.3.2 C, D, E, F, the carboxypeptidase is produced by submerged fermentation of a genetically modified *Aspergillus oryzae* strain.

The safety of the production organism and the enzyme product has been thoroughly assessed:

- The production organism has a long history of safe use as production strain for food-grade enzyme preparations and is known not to produce any toxic metabolites.
- The genetic modifications in the production organism are well-characterised and safe and the recombinant DNA is stably integrated into the production organism and unlikely to pose a safety concern.
- The enzyme preparation complies with international specifications ensuring absence of contamination by toxic substances or noxious microorganisms
- Sequence homology assessment to known allergens and toxins shows that oral intake of the carboxypeptidase does not pose food allergenic or toxic concern.
- Two mutagenicity studies *in vitro* showed no evidence of genotoxic potential of the enzyme preparation.
- An oral feeding study in rats for 13-weeks showed that all dose levels were generally well tolerated and no evidence of toxicity.

F Assessment procedure

Because the application is for a new source organism for a new enzyme in the Code, it is considered appropriate that the assessment procedure is characterised as "General Procedure, Level 1".

G Confidential commercial information (CCI)

Detailed information on the raw materials used in production of the enzyme preparation and construction and characteristics of the genetically modified production strain are provided in Appendix 4 and 6, respectively. Summaries of the information are given in section A.4 and

3.3.2 E. The formal request for treatment of selected parts of Appendix 4 and 6 as confidential commercial information (CCI) is included as Appendix 1.1.

H Other confidential information

Apart from the selected parts of Appendix 4 and 6 identified as confidential commercial information (CCI), no other information is requested to be treated as confidential.

I Exclusive capturable commercial benefit (ECCB)

This application is not expected to confer an Exclusive Capturable Commercial Benefit.

J International and other national standards

J.1 International Standards

Use of enzymes as processing aids for food production is not restricted by any Codex Alimentarius Commission (Codex) Standards.

J.2 Other national standards or regulations

With few exceptions on national, commodity standards, use of enzymes as processing aids for food production is in general not restricted by standards or regulations in other countries.

K Statutory declaration

The Statutory Declaration is provided as a separate document together with this submission.

L Checklist

This application concerns an enzyme product intended to be used as a processing aid. Therefore, the relevant documentation according to the Application Handbook from Food Standards Australia New Zealand as of 1 July 2019, are the following sections:

- Section 3.1.1 – General requirements
- Section 3.3.2 – Processing aids, subsections A, C, D, E, F

Accordingly, the checklist for General requirements as well as the Processing aids part of the checklist for applications for substances added to food was used and is included as Appendix 1.2 and 1.3.

CHAPTER 3.3, GUIDELINES FOR APPLICATIONS FOR SUBSTANCES ADDED TO FOOD

3.3.2 PROCESSING AIDS

The carboxypeptidase enzyme preparation described in this application is representative of the commercial food enzyme product for which approval is sought.

A Technical information on the processing aid

A.1 Information on the type of processing aid

The carboxypeptidase enzyme preparation belongs to the category of processing aids described in Schedule 18—Processing aids.

The carboxypeptidase enzyme preparation is to be used in the food industry as a processing aid during the processing of raw materials containing proteins. Carboxypeptidases hydrolyse proteins into shorter proteins/peptides and free amino acids.

The carboxypeptidase enzyme preparation is used in, but not limited to, the following food manufacturing processes:

- In protein hydrolysis of peptide bonds in manufacturing and/or processing of proteins, yeast, and flavouring, the carboxypeptidase is used to hydrolyse proteins into shorter proteins/peptides and free amino acids.
- In the manufacture of bakery products, the carboxypeptidase partially hydrolyses the gluten network in the dough.
- In brewing, the carboxypeptidase increases the amount of free amino nitrogen by hydrolysis of proteins present in the raw materials.

The highest dosage of the carboxypeptidase during a food manufacturing process is in protein hydrolysis, where dosages up to 40,000 CPDU(A) per kg protein concentrate or isolate are used.

A.2 Information on the identity of the processing aid

A.2.1 Enzyme

Generic name	carboxypeptidase
IUBMC nomenclature	carboxypeptidase D
IUBMC No.	EC 3.4.16.6
Cas No.	153967-26-1

A.2.2 Enzyme preparation

The enzyme concentrate is formulated into a final enzyme preparation. The enzyme concentrate may be intended for a single enzyme preparation or a blend with other food enzymes and formulated as a liquid product depending on the characteristics of the intended food process in which it will be used.

The typical composition of the enzyme concentrate is:

Enzyme solids (TOS)[1]	approx. 4.0 %
Sucrose	approx. 30.0 %
Sodium chloride	approx. 10.0 %
Potassium sorbate	approx. 0.2 %
Water	approx. 55.8 %

The enzyme concentrate is standardised in carboxypeptidase units to an activity of 890 CPDU(A)/g. The Novozymes A/S method used to determine the CPDU(A) activity is enclosed in Appendix 3.1.

Briefly, the carboxypeptidase hydrolyses the peptide bond between a peptide and a chromophore. This reaction produces an absorption decrease at 340 nm, which is proportional to the enzyme activity.

[1] TOS = Total Organic Solids, defined as: 100% - water - ash - diluents

A.2.3 Host organism

The production strain was developed from the *Aspergillus oryzae* A1560 (synonym IFO 4177) cell lineage, which was obtained from Institute for Fermentation Osaka (IFO). The A1560 cell lineage has a long history of safe use at Novozymes A/S for production of food enzymes and has given rise to a number of food enzyme production strains, which are used for production of previously evaluated and regulatory approved food enzymes. The taxonomic classification of the strain is:

Division	Ascomycota
Class	Eurotiomycetes
Order	Eurotiales
Family	Trichocomaceae
Genus	*Aspergillus*
Species	*Aspergillus oryzae*

For a more detailed description of the host organism and the genetic modifications, please see section 3.3.2 E.

A.2.4 Donor organism

The donor for the carboxypeptidase gene is *Aspergillus oryzae*.

For a more detailed description of the donor and the donor gene, please see section 3.3.2 E.

A.3 Information on the chemical and physical properties of the processing aid

The enzyme is a carboxypeptidase D (EC 3.4.16.6), commonly known as carboxypeptidase. Carboxypeptidases catalyse the hydrolysis of peptide bonds, preferably before C-terminal arginine and lysine residues.

The enzyme preparation is available as liquid product.

The food enzyme object of the present application is not added to final foodstuffs but used as a processing aid during food manufacturing.

No reaction products, which could not be considered normal constituents of the diet, are formed during the production or storage of the enzyme-treated food.

A.4 Manufacturing process

The manufacturing process is composed of a fermentation process, a purification process, a formulation process and finally a quality control of the finished product, as outlined by Aunstrup et al. (1979). This section describes the processes used in manufacturing of the carboxypeptidase enzyme product.

The enzyme preparation is manufactured in accordance with current Good Manufacturing Practices (Appendix 4.1). The quality management system used in the manufacturing process complies with ISO 9001:2015 (Appendix 4.2).

The raw materials are of food-grade quality and have been subjected to appropriate analysis to ensure their conformity with the specifications.

A.4.1 Fermentation

The carboxypeptidase is produced by submerged fed-batch pure culture fermentation of the genetically modified strain of *Aspergillus oryzae*, described in section 3.3.2 E.

A.4.1.1 Raw materials for fermentation

The production strain is grown in a medium consisting of compounds providing an adequate supply of carbon and nitrogen as well as minerals and vitamins necessary for growth. Furthermore, acids and bases for the adjustment of the pH and processing aids (e.g. antifoaming agents) are used during fermentation. The choice of raw materials used in the fermentation process (the feed, the seed fermenter, the main fermenter and dosing) is given in the confidential parts of Appendix 4.3.

A.4.1.2 Hygienic precautions

All equipment is designed and constructed to prevent contamination by foreign microorganisms.

All valves and connections not in use for the fermentation are sealed by steam at more than 120 °C.

After sterilization a positive pressure of more than 0.2 atmosphere is maintained in the fermentation tank.

The air used for aeration is sterilised by passing through a sterile filter. The inside of each fermentation tank is cleaned between fermentations by means of a high-pressure water jet and inspected after the cleaning procedures have been completed.

A.4.1.3 Preparation of the inoculum

The inoculum flask containing the prepared medium is autoclaved and checked. Only approved flasks are used for inoculation.

The stock culture suspension is injected aseptically into the inoculum flask and spread onto the medium in the flask. Once growth has taken place in the inoculum flask (typically after a few days at 30°C), the following operations are performed:

- Strain identity and traceability: ampoule number is registered
- Microbial purity: a sample from the inoculum flask is controlled microscopically for absence of microbial contaminants.

When sufficient amount of biomass is obtained and when the microbiological analyses are approved, the inoculum flask can be used for inoculating the seed fermenter.

A.4.1.4 The seed fermentation

The raw materials for the fermentation medium are mixed with water in a mixing tank. The medium is transferred to the seed fermenter and heat sterilised (e.g. 120 °C/60 min).

The seed fermentation tank is inoculated by transferring aseptically a suspension of cells from the inoculum flask.

The seed fermentation is run aerobically (sterile airflow), under agitation. The overpressure is kept above 0.2 atmosphere at all times, to prevent contamination.

Once a sufficient amount of biomass has developed, microbiological analyses are performed to ensure absence of contamination. The seed fermentation can then be transferred to the main fermentation tank.

A.4.1.5 The main fermentation

The raw materials for the medium are mixed with water in a mixing tank. The medium is transferred to the main fermenter and heat sterilised (e.g. 120 °C/60 min). If necessary, the pH is adjusted after sterilization, with sterile pH adjustment solutions.

The fermentation in the main tank is run as normal submerged fed-batch fermentation.

The main fermentation is run aerobically (sterile airflow), under vigorous agitation. The overpressure is kept above 0.2 atmosphere at all times, to prevent contamination. The fermentation is run at a well-defined temperature.

Fresh medium is added aseptically when the pH increases above its set point, and the dissolved oxygen concentration rises. The feed rate is adjusted so that there is no accumulation of carbohydrates.

Other parameters are measured at regular intervals

- refractive index
- enzyme productivity
- residual glucose
- residual ammonia

Samples are also taken at regular intervals to check absence of microbial contamination.

A.4.2 Recovery

The recovery process is a multi-step operation designed to separate the enzyme from the microbial biomass and partially purify, concentrate, and stabilize the food enzyme.

The steps of this process involve a series of typical unit operations:

- pre-treatment
- primary separation
- filtration
- concentration
- evaporation
- preservation and stabilization

A.4.2.1 Raw materials for recovery

The choice of raw materials used during recovery is given in the confidential parts of Appendix 4.3.

A.4.2.2 Pre-treatment

To facilitate the separation, flocculants are used in a pH-controlled process.

A.4.2.3 Primary separation

The cell mass and other solids are separated from the broth by well-established techniques such as pre-coat vacuum drum filtration or centrifugation.

The primary separation is performed at well-defined pH and temperature range.

A.4.2.4 Filtration

For removal of residual cells of the production strain and as a general precaution against microbial degradation, filtration on dedicated germ filtration media is applied. Pre-filtration is included when needed.

The filtrations are performed at well-defined pH and temperature intervals, and result in an enzyme concentrate solution free of the production strain and insoluble substrate components from the fermentation.

A.4.2.5 Concentration

Ultrafiltration and/or evaporation are applied for concentration and further purification. The ultrafiltration is applied to fractionate high molecular weight components (enzymes) from low molecular weight components and is used to increase the activity/dry matter ratio. Evaporation is used to increase the activity while maintaining the activity/dry matter ratio.

The pH and temperature are controlled during the concentration step, which is performed until the desired activity and activity/dry matter ratio has been obtained.

A.4.2.6 Evaporation

Evaporation is performed to remove water and increase the refractive index. The concentration is run at 0-45 °C and the refractive index is controlled during the concentration step to ensure that the dry matter content is within a given range.

A.4.2.7 Preservation and stabilization

For enzymatic, physical and microbial stabilization sucrose, sodium chloride as well as potassium sorbate are added to the enzyme concentrate.

A.4.2.8 Process control

Apart from the process controls performed during the various fermentation steps and described above, the following microbial controls are also performed.

Samples are withdrawn from both the seed fermenter and the main fermenter:

- before inoculation

- at regular interval during cultivation
- before transfer/harvest

The samples during all steps are examined by:

- microscopy
- plating culture broth on a nutrient agar and incubating for 24-48 hours

Growth characteristics are observed macroscopically and microscopically.

During the microbiological control steps, the number of foreign microorganisms should be insignificant. The fermentation parameters, i.e. enzyme activity, temperature and oxygen as well as pH are also monitored closely. A deviation from the normal course of the fermentation may signal a contamination.

If a significant contamination develops, the fermentation is terminated. The fermentation is regarded as "significantly contaminated" if two independent samples show presence of contaminating organisms after growth on nutrient agar.

Any contaminated fermentation is rejected for enzyme preparations to be used in a food-grade application.

A.5 Specification for identity and purity

The carboxypeptidase enzyme product complies with the purity criteria recommended for Enzyme Preparations in Food, Food Chemicals Codex, 11th edition, 2018.

In addition to this, the carboxypeptidase enzyme product also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications.

Analytical data for three representative batches of the carboxypeptidase enzyme preparation are shown in (Table 1). These data show compliance with the purity criteria of the specification.

Table 1: Analytical data for three representative enzyme product batches

Control parameter	Specification	Batch 1	Batch 2	Batch 3
Lead (mg/kg)	≤ 2	ND (LOD < 0.5)	ND (LOD < 0.5)	ND (LOD < 0.5)
Arsenic (mg/kg)	≤ 1	ND (LOD < 0.3)	ND (LOD < 0.3)	ND (LOD < 0.3)
Cadmium (mg/kg)	≤ 1	ND (LOD < 0.05)	ND (LOD < 0.05)	ND (LOD < 0.05)
Mercury (mg/kg)	≤ 1	ND (LOD < 0.05)	ND (LOD < 0.05)	ND (LOD < 0.05)
Total coliforms (CFU/g)	≤ 30	<4	<4	<4
Enteropathogenic *Escherichia coli* (CFU/ 25 g)	ND	ND	ND	ND
***Salmonella* spp. (CFU/ 25 g)**	ND	ND	ND	ND
Antimicrobial activity	ND	ND	ND	ND
Aflatoxin B1 (mg/kg)		ND (LOD < 0.0003)	ND (LOD < 0.0003)	ND (LOD < 0.0003)
Beta-nitro propionic acid (mg/kg)		ND (LOD* < 0.18)	ND (LOD* < 0.18)	ND (LOD* < 0.18)
Cyclopiazonic acid (mg/kg)		ND (LOD < 0.003)	ND (LOD < 0.003)	ND (LOD < 0.003)
Kojic acid (mg/kg)		ND (LOD* < 0.029)	ND (LOD* < 0.03)	ND (LOD* < 0.03)

ND: not detected; LOD: limit of detection; CFU: colony-forming unit (*: LOD is matrix-dependent); CFU: colony forming unit

The methods of analysis used to determine compliance with the specifications are enclosed (Appendix 3).

The carboxypeptidase enzyme preparation is available as a liquid enzyme concentrate. The concentrate is standardised in carboxypeptidase units (CPDU(A)/g; Appendix 3.1). The preparation does not contain known food allergens (Appendix 2.1).

A.6 Analytical method for detection

The carboxypeptidase enzyme preparation is to be used in the food industry as a processing aid. This information is not required in the case of an enzymatic processing aid.

B Information related to the safety of a chemical processing aid

Not applicable – this application does not concern a chemical processing aid.

C Information related to the safety of an enzyme processing aid

C.1 General information on the use of the enzyme as a food processing aid in other countries

The enzyme can be used as processing aid during processing of protein-containing raw materials in a range of countries, where there are no restrictions of the use of enzyme processing aids.

This application describes a newly developed enzyme. As a result, it has not been assessed by other authorities or external expert groups.

C.2 Information on the potential toxicity of the enzyme processing aid

(a) Information on the enzyme's prior history of human consumption and/or its similarity to proteins with a history of safe human consumption

A wide variety of enzymes are used in food processing. Enzymes, including proteases and peptidases, have a long history of use in food (Pariza and Foster, 1983 and Pariza and Johnson, 2001).

Proteases have been used extensively for food processing for many centuries (Sumantha et al., 2006). They are essential to produce cheese and they are also used in brewing, baking, and flavour enhancement processes (Pariza and Foster, 1983; Sumantha et al., 2006; Singh et al., 2016). Protease enzyme preparations from various sources are widely authorised in, e.g. Australia and New Zealand, Brazil, Canada, China, Denmark, France, Mexico.

(b) Information on any significant similarity between the amino acid sequence of the enzyme and that of known protein toxins

A sequence homology assessment of the carboxypeptidase enzyme to known toxins was conducted. The amino acid sequence of the carboxypeptidase provided in Appendix 6.4 was used as input for the search. No homologies to known toxins were found. The complete search report is enclosed in Appendix 5.1.

Furthermore, safety studies as described below were performed on representative batches (PPJ55674 and PPJ60566) that were produced according to the description given in section 3.3.2 A.4, omitting stabilization and standardization. A summary of the safety studies is enclosed in Appendix 5.2.

The following studies were performed:

- Ames Test. Test for mutagenic activity on batch PPJ55674 (Appendix 5.3)
- *In vitro* micronuclei test on batch PPJ55674 (Appendix 5.4)
- Subchronic (13 week) oral toxicity study in rats on batch PPJ60566 (Appendix 5.5)

The main conclusions of the safety studies can be summarised as follows:

- Carboxypeptidase PPJ55674 did not induce gene mutations in bacteria either in the presence or absence of metabolic activation (S-9) when tested under the conditions employed in this study.
- Carboxypeptidase PPJ55674 did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the presence or absence of an aroclor induced rat liver metabolic activation system (S-9).
- Oral administration of Carboxypeptidase PPJ60566 to Sprague-Dawley rats at doses up to 100 % of the tox test batch (2220 mg TOS/kg bw/day for 13 weeks was well-tolerated and did not cause any adverse change. The NOAEL was considered to be 100 % of the tox test batch (equivalent to 2220 mg TOS/kg bw/day).

Based on the present toxicity data it can be concluded that the carboxypeptidase enzyme preparation, represented by batches PPJ55674 and PPJ60566, exhibits no toxicological effects under the experimental conditions described.

C.3 Information on the potential allergenicity of the enzyme processing aid

(a) Information of the source of the enzyme processing aid

The carboxypeptidase enzyme is produced by an *Aspergillus oryzae* microorganism expressing the carboxypeptidase from *Aspergillus oryzae*. *Aspergillus oryzae* is ubiquitous in the environment and in general considered as a non-pathogenic fungus (see Section 3.3.2 D).

(b) Analysis of similarity between the amino acid sequence of the enzyme and that of known allergens

Enzymes have a long history of safe use in food, with no indication of adverse effects or reactions. Moreover a wide variety of enzyme classes (and structures) are naturally present in food.

The allergenicity potential of enzymes was studied by Bindslev-Jensen et al (2006) and reported in the publication: “Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry". The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and protein engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.

Additionally, food enzymes are used in small amounts during food processing resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al, 2008).

A sequence homology assessment of the carboxypeptidase enzyme to known allergens was conducted (Appendix 5.1). The amino acid sequence of the carboxypeptidase provided in Appendix 6.4 was used as input for the search. The carboxypeptidase was compared to allergens from the FARRP allergen protein database (http://www.allergenonline.org).

The sequence homology analyses identified sequence homology between the carboxytpeptidase and P08819, a serine carboxypeptidase originating from wheat (*Triticum aestivum*), of 50.8 % and Api m 9.0101, a serine carboxypeptidase produced by honey bee (*Apis mellifera*), of 53.6 % (Appendix 5). No homology to other allergens was found.

The first hit shows a 50.8 % sequence homology to P08819, a serine carboxypeptidase originating from wheat (*Triticum aestivum*). The full length identity is 22.8 % which is low and thus not considered problematic. This carboxypeptidase is a suspected allergen and is included in Allergen.org; however, it is registered without being assigned a name and included on the basis of in vitro IgE evidence but without any relevant biological tests (http://www.allergenonline.org).

The second hit has a 53.6 % sequence homology to Api m 9.0101, a serine carboxypeptidase produced by honey bee (*Apis mellifera*). The identity across the full length is 22.9% which is also low and thus not considered problematic. Api m 9 is a honey bee serine carboxypeptidase expressed in the venom duct. No literature on Api m 9 and allergy was found and it appears to be identified as an allergen based only on sequence homology to other peptidases. The only route of allergen exposure described in Allergen.org is by injection.

There is compelling evidence that majority of adults affected by food-induced occupational asthma can ingest the allergen without symptoms, suggesting that inhalation is not likely to result in food allergy (Armentia et al., 2009).

In addition, the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products (Dauvrin et al, 1998). In this paper, Dauvrin et al. conclude that enzyme exposure by ingestion, in opposition to exposure by inhalation, is extremely unlikely to lead to sensitization. Only one single case has been reported in the literature and this case was not verified as a bona fide oral sensitization to enzymes in food.

This is backed up by a study using the generally recognized guidelines for food allergy diagnosis (skin prick test, specific serum IgE and DBPCFC). This study included 400 patients with diagnosed allergy to one or more of inhalation allergens, food allergens, bee or wasp allergens. The study concluded that no cases of IgE-mediated food allergy to commercial enzymes could be found. There were further no indications of cross-reactivity between the tested enzymes used in food and the main known allergens represented by the patients included in the study (Bindslev-Jensen et al., 2006).

On the basis of the available evidence it is concluded that oral intake of the carboxypeptidase is not anticipated to pose any food allergenic concern.

C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available

This application describes a newly developed enzyme. As a result, it has not been assessed by other authorities or external expert groups.

D Additional information related to the safety of an enzyme processing aid derived from a microorganism

D.1 Information on the source microorganism

The carboxypeptidase enzyme is produced by an *Aspergillus oryzae* microorganism expressing the carboxypeptidase from *Aspergillus oryzae*. The *Aspergillus oryzae* host strain A1560 (synonym IFO 4177) was obtained from Institute for Fermentation Osaka (IFO). The A1560 cell lineage has a long history of safe use at Novozymes A/S for production of food enzymes and has given rise to a number of food enzyme production strains, which are used for production of previously evaluated and regulatory approved food enzymes.

The carboxypeptidase production strain is a non-pathogenic, non-toxigenic, genetically modified *Aspergillus oryzae* strain. The production strain is marker-free, and it does not produce secondary metabolites of toxicological concern to humans as explained in Section E 1.3, Section A.5 and Appendix 6.1.

D.2 Information on the pathogenicity and toxicity of the source microorganism

Aspergillus oryzae is a filamentous fungus recognised to be non-pathogenic for humans, animals and plants.

Aspergillus oryzae is classified as a group 1 microorganism according to EU Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work. A group 1 biological agent means one that is unlikely to cause human disease.

As all other fungal species, *Aspergillus oryzae* has not been assigned the QPS status as defined by EFSA's Scientific committee in their Opinion published 6 December 2007. However, *Aspergillus oryzae* as a species has been used safely for the production of food enzymes world-wide for more than 30 years.

Barbesgaard et al. (1992) reviewed the safety of *Aspergillus oryzae* and describe it as having a very long history of safe industrial use, being widely distributed in nature, and being commonly used for production of food enzymes.

Enzyme preparations containing *Aspergillus oryzae* or containing enzymes produced by *Aspergillus oryzae* have been used for hundreds of years in the manufacture of Koji and Miso. Furthermore, enzymes from *Aspergillus oryzae* have been used extensively for decades in the Western part of the world for the production of a variety of foods (including syrups, alcohol, fruit juices, meat tendering, brewing and baking products) and use can be dated back to at least 1918 (Beckhorn, 1965; Cook et al., 1994).

Enzymes produced by *Aspergillus oryzae* (a.o. alpha-amylase and protease[2], asparaginase and phospholipase[3], laccase[4]) have been positively evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Carbohydrase, protease, and lipase enzyme preparations from *Aspergillus oryzae* are included in the GRAS petition 3G0016 (filed April 12th, 1973) that FDA on request from the Enzyme Technical Association (ETA) converted into separate GRAS Notices[5] (GRN 90 and GRN 113). Based on the information provided by ETA, as well as the information in GRP 3G0016 and other information available to FDA, the agency did not question the conclusion that enzyme preparations from *Aspergillus oryzae* are GRAS under the intended conditions of use. Analogous conclusions were drawn in GRAS Notices GRN 34, 43, 75, 103, 106, 142, and 201 which all describe food enzymes produced by *Aspergillus oryzae* strains originating from the Novozymes *Aspergillus oryzae* A1560 strain line.

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[2] http://www.inchem.org/documents/jecfa/jecmono/v22je03.htm
[3] http://www.inchem.org/documents/jecfa/jecmono/v59je01.pdf
[4] http://www.inchem.org/documents/jecfa/jecmono/v52je06.htm
[5] GRAS Notice Inventory: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices

Aspergillus oryzae as a species is known to have the potential to produce the secondary metabolites cyclopiazonic acid (CPA), beta-nitropropionic acid (NPA), and kojic acid (KA) (Frisvad et al., 2018). However, as described in Appendix 6, the production strain is incapable of producing CPA, and it does not produce other species relevant secondary metabolites under conditions employed for enzyme production as demonstrated by analytical data presented in Table 1.

Overall, it can be concluded that *Aspergillus oryzae* is widely accepted as a non-pathogenic organism and has a long history of safe use in food and food enzyme production.

D.3 Information on the genetic stability of the source organism

The inserted recombinant DNA is genetically stable during fermentation, as the inserted DNA is integrated into the chromosome.

Stability of the introduced DNA sequences was analysed using phenotypic characteristics of the production strain, i.e. enzyme activity and protein synthesis.

For a more detailed description of the strain construction and characteristics, please see section 3.3.2 E.

E Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism

E.1 Information on the methods used in the genetic modification of the source organism

This section contains summarised information on the modifications of the host strain, on the content and nature of the introduced DNA and on the construction of the final production strain, as well as the stability of the inserted gene. The detailed information is provided in the confidential Appendix 6.

E.1.1 Host organism

The production strain was developed from the *Aspergillus oryzae* A1560 (synonym IFO 4177) cell lineage, which was obtained from Institute for Fermentation Osaka (IFO). The A1560 cell lineage has a long history of safe use at Novozymes A/S for production of food enzymes and has given rise to a number of food enzyme production strains, which are used for production of previously evaluated and regulatory approved food enzymes. The taxonomic classification of the strain is:

Division Ascomycota

Class Eurotiomycetes

Order Eurotiales

Family Trichocomaceae

Genus *Aspergillus*

Species *Aspergillus oryzae*

The recipient strain used in the construction of the *Aspergillus oryzae* production strain, was derived from the parental strain through a combination of classical mutagenesis/selection and GM-steps. These steps were carried out in order to simplify purification, enhance product stability and increase the safety of the strain.

E.1.2 Introduced DNA

The vector used to transform the *Aspergillus oryzae* recipient strain is based on a *Escherichia coli* standard vector. No elements of the vector are left in the production strain. The vector contains the carboxypeptidase expression cassette consisting of an *Aspergillus* promoter, the coding sequence for carboxypeptidase from *Aspergillus oryzae* and an *Aspergillus* terminator.

E.1.3 Construction of the Recombinant Microorganism

The *Aspergillus oryzae* production strain was constructed from the recipient strain through the following steps:

1. The carboxypeptidase expression cassette was integrated at specific integration sites present in the recipient strain.

2. A transformant was screened for rapid growth and high carboxypeptidase activity leading to the final production strain.

E.1.4 Antibiotic Resistance Gene

No functional antibiotic resistance genes were left in the strain as a result of the genetic modifications as shown by genome sequence analysis.

E.1.5 Stability of the Introduced Genetic Sequences

The transforming DNA is stably integrated into the *Aspergillus oryzae* chromosome and, as such, is poorly mobilised for genetic transfer to other organisms and is mitotically stable.

Stability of the introduced DNA sequence was analysed using phenotypic characteristics of the production strain, i.e. enzyme activity and protein synthesis. Further details can be found in Appendix 6.4.

F Information related to the dietary exposure to the processing aid

F.1 A list of foods or food groups likely to contain the processing aid or its metabolites

The carboxypeptidase preparation is used as a processing aid during the manufacture of protein-based products. Carboxypeptidases hydrolyse proteins into shorter proteins/peptides and free amino acids.

F.2 The levels of residues of the processing aid or its metabolites for each food or food group

The carboxypeptidase enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following GMP.

The enzyme is used in several processes for processing raw materials containing proteins.

- In protein hydrolysis of peptide bonds in manufacturing and/or processing of proteins, yeast, and flavouring, the carboxypeptidase is used to hydrolyse proteins into shorter proteins/peptides and free amino acids.
- In the manufacture of bakery products, the carboxypeptidase partially hydrolyses the gluten network in the dough.
- In brewing, the carboxypeptidase increases the amount of free amino nitrogen by hydrolysis of proteins present in the raw materials.

Use level

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following GMP.

The conditions of use of the carboxypeptidase preparation during food processing do not only depend on the type of application, but also on the food production process of each individual food manufacturer. In order to ensure optimal effectiveness of the enzyme at an acceptable economic cost the dosage, reaction time, process conditions and processing steps are adjusted.

The highest dosage given for solid food is 40,000 CPDU(A) per kg protein concentrate or isolate. This corresponds to 44.9 g of carboxypeptidase enzyme preparation per kg protein concentrate or isolate equivalent to 1,796 mg TOS per kg protein concentrate or isolate.

The highest dosage given for liquids is 750 CPDU(A) per kg cereals (malted or not). This corresponds to 0.84 g of carboxypeptidase enzyme preparation per kg cereals (malted or not) equivalent to 33.6 mg TOS per kg cereals (malted or not).

Enzyme residues in the Final Food

The carboxypeptidase preparation is used in processing of raw materials containing proteins for the hydrolysis proteins into shorter proteins/peptides and free amino acids. The enzyme is denatured by heat during processing.

F.2.1 Estimates of human consumption

Method used for the dietary exposure assessment

An exposure assessment according to the Budget Method (Hansen, 1966; Douglass et al., 1997; ILSI, 1997) has been performed, as the processed proteins are used as an ingredient in a variety of food products and beverages.

Budget Method

Overall, the human exposure to the carboxypeptidase will be negligible because the enzyme preparation is used as a processing aid and in low dosages.

The Budget Method assumptions represent a “maximum worst case” situation of human consumption, in which the food enzyme object of the present application would be used at its maximum recommended dosages in all processed food and all processed beverages and not only in those food and drink processes described in Section F.2.

It is also supposed that the totality of the food enzyme will end up in the final food. This assumption is exaggerated since the enzyme protein and the other substances resulting from the fermentation are diluted or removed in certain processing steps.

Therefore the safety margin calculation derived from this method is highly conservative.

Assumptions in the Budget Method

Solids	The maximum energy intake over the course of a lifetime is 50 kcal/kg body weight/day. 50 kcal corresponds to 25 g foods.

	Therefore, adults ingest 25 g foods per kg body weight per day. Assuming that 50% of the food is processed food, the daily consumption will be 12.5 g processed foods per kg body weight. It is further assumed that, in average, all processed food contains 25% proteins (or protein-derived) dry matter = 3.12 g protein-derived dry matter per kg body weight per day.
Liquids	The maximum intake of liquids (other than milk) is 100 ml/kg body weight/day. Assuming that 25% of the non-milk beverages is processed, the daily consumption will be 25 ml processed beverages per kg body weight. It is further assumed that all processed beverages contain 12% protein hydrolysates = 3.0 g protein-derived dry matter per kg body weight per day. It is assumed that the densities of the beverages are ~ 1.

TMDI (Total amount of dietary intake) calculation

Solid food

The highest dosage given for solid food is 40,000 CPDU(A) per kg protein concentrate or isolate, corresponding to 1,796 mg TOS per kg protein concentrate or isolate (cf. Section 3.3.2 A.2.2).

Based on this, 3.12 g protein-derived dry matter in solid food will maximally contain:

1,796 mg TOS per kg / 1000 g per kg x 3.12 g = 5.6 mg TOS

Liquids

The highest dosage given for liquids is 750 CPDU(A) per kg protein concentrate or isolate, corresponding to 33.6 mg TOS per kg protein concentrate or isolate (cf. Section 3.3.2 A.2.2).

Based on this, 3.0 g protein-derived dry matter in liquids will maximally contain:

33.6 mg TOS per kg / 1000 g per kg x 3.0 g = 0.1 mg TOS

Total TMDI of protein-derived solid foods and liquids

5.6 mg TOS + 0.1 mg TOS = 5.7 mg TOS

F.2.2. Safety Margin Calculation

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption (TMDI). The NOAEL dose level in the 13 weeks oral toxicity study in rats was concluded to be and 2,220 mg TOS/kg bw/day (cf. Section 3.3.2 C 2).

The estimated human consumption is 5.7 mg TOS/kg/day

The safety margin can thus be calculated to be and 2,220/5.7 = 389.

F.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption

Not relevant.

F.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

It is assumed that all raw materials containing proteins are processed using the carboxypeptidase object of this submission as a processing aid at the highest recommended dosage.

F.5 Information relating to the levels of residues in foods in other countries

As described in F.2.1 above, a "worst case" calculation is made assuming that all organic matter originating from the enzyme is retained in the processed food product. The dietary exposure is estimated using the Budget Method, as the processed proteins are used as an ingredient in a variety of food products.

F.6 For foods where consumption has changed in recent years, information on likely current food consumption

No significant changes in recent years are observed.

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LIST OF APPENDICES

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